Regulation of immune responses via co-signaling receptors in human and animal immunity models

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ΠΕΡΙΛΗΨΗ

Ο Συστηματικός Ερυθματώδης Λύκος (ΣΕΛ) είναι ένα συστεμικό αυτοάνοσο νόσημα το οποίο χαρακτηρίζεται από την ανίχνευση αντι-πυρηνικών αυτό-αντισωμάτων και τη διαδοχή περιόδων έξαρσης των συμπτωμάτων και περιόδων ύφεσης όπου τα συμπτώματα υποχωρούν. Η ύπαρξη αυτό-αντισωμάτων στον ορό των ασθενών ακόμα και πριν την εμφάνιση συμπτωμάτων, καθώς και in vivo μελέτες σε ζωικά μοντέλα του ΣΕΛ μαρτυρούν ότι ακρογωνιαίος λίθος της παθογένειας του ΣΕΛ είναι τα Β λεμφοκύτταρα.

Έχει δειχθεί ότι η ενεργοποίηση των Β λεμφοκυττάρων απαιτεί την ταυτόχρονη σηματοδότηση από τον υποδοχέα των Β λεμφοκυττάρων (BCR) και ταυτόχρονη σηματοδότηση από έναν υποδοχέα κυτταροκινών και συν-διεγερτικούς υποδοχείς. Η σηματοδότηση μέσω κυτταροκινών και συν-διεγερτών προέρχεται από τα Τα λεμφοκύτταρα βοηθούς. Μελέτες έχουν δείξει ότι υπάρχει ένα πλήθος συν-διεγερτικών υποδοχέων οι οποίοι με βάση την αλληλουχία των αμινοξέων τους χωρίζονται σε 2 υπεροικογένειες, την υπεροικογένεια των ανοσοσφαιρινών (Ig superfamily) και την υπεροικογένεια του Παράγοντα Νέκρωσης Όγκων (TNF superfamily). Με βάση τη λειτουργία τους οι συν-διεγερτικοί υποδοχείς χωρίζονται σε συν-ενεργοποιητές (co-stimulators) και συν-καταστολείς (co-inhibitors).

Η παρούσα μελέτη είχε ως στόχο τη διερεύνηση του ρόλου του συν-διεγερτικού υποδοχέα HVEM και των υποδοχέων στην επιφάνεια των Β λεμφοκυττάρων που τον αναγνωρίζουν (BTLA, LIGHT) στην ενεργοποίηση των Β λεμφοκυττάρων τόσο σε φυσιολογικές όσο και σε αυτοάνοσες αποκρίσεις. Τα αποτελέσματα έδειξαν ότι η τριανδρία HVME/BTLA/LIGHT επηρεάζει με διαφορετικό τρόπο την ενεργοποίηση των Β λεμφοκυττάρων σε χρονική κλίμακα. Βραχυπρόθεσμες καλλιέργειες έδειξαν μείωση της ενεργοποίησης των Β λεμφοκυττάρων, ενώ μακροπρόθεσμες καλλιέργειες δείχνουν αναστροφή του φαινομένου και πιο έντονη ενεργοποίηση των Β λεμφοκυττάρων παρουσία του HVEM. Επιπλέον, σηματοδότηση από τον HVEM φαίνεται να προστατεύει τα Β λεμφοκύτταρα από τον προγραμματισμένο κυτταρικό θάνατο, ενώ παράλληλα καθυστερεί τον πολλαπλασιασμό τους.

Συνοψίζοντας, τα παρόντα αποτελέσματα καταδεικνύουν ένα διττό ρόλο του HVEM και των υποδοχέων του κατά την ενεργοποίηση των Β λεμφοκυττάρων. Περεταίρω πειράματα είναι απαραίτητα προκειμένου να μελετηθεί σε βάθος το σηματοδοτικό τρίδυμο HVEM/BTLA/LIGHT και η συμβολή του στην ενεργοποίηση των Β λεμφοκυττάρων τόσο σε περιπτώσεις ανοσολογικής απόκρισης όσο και σε περιπτώσεις αυτοανοσίας.

ΕΥΧΑΡΙΣΤΙΕΣ

Ολοκληρώνοντας τη διπλωματική μου εργασία θα ήθελα να ευχαριστήσω όλους αυτούς που είτε εν γνώσει τους είτε όχι με βοήθησαν στην ολοκλήρωσή της. Αρχικά θα ήθελα να ευχαριστήσω τον κ. Μπερτσιά τόσο για την επιλογή μου ως μεταπτυχιακό φοιτητή του εργαστηρίου όσο και για την άρτια συνεργασία μου μαζί του τον τελευταίο χρόνο. Επιπλέον θα ήθελα να ευχαριστήσω όλα τα μέλη του εργαστήριου «Αυτοανοσίας και Φλεγμονής», ιδιαίτερα τις συνεργάτιδες ‘επί του πάγκου’ για την βοήθεια, τη στήριξή και την υπομονή τους εντός και εκτός εργαστηρίου τον τελευταίο αυτό χρόνο. Επιπλέον, θα ήθελα να ευχαριστήσω τους γονείς μου, τον αδερφό μου Κωνσταντίνο και τους φίλους μου για την οικονομική και ψυχολογική στήριξή τους και την ανεξάντλητη υπομονή που έδειξαν απέναντί μου. Τέλος, θα ήθελα να ευχαριστήσω όλα τα μέλη της Ρευματολογικής Κλινικής του Πανεπιστημιακού Νοσοκομείου Ηρακλείου καθώς και το προσωπικό του τμήματος αιμοδοσίας του Πανεπιστημιακού Νοσοκομείου Ηρακλείου, χωρίς τη συνεργασία των οποίων η παρούσα εργασία δεν θα μπορούσε ποτέ να ολοκληρωθεί.

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A. INTRODUCTION

A.1 Systemic Lupus Erythematosus

SLE is a systemic, inflammatory, autoimmune disease characterized by the presence of anti-nuclear antibodies in the serum of patients and an oscillation of disease symptoms with periods of increased disease activity (flares) followed by quiescent periods (remissions) and can cause severe damage to a variety of organs and systems such as the brain; kidney and blood vessels (Bertsias GK et al. 2010). Interestingly, lupus affects mostly females (9:1 female to male ratio) with blacks, Asians, Hispanics and Native Americans showing higher susceptibility than whites (Ippolito et al. 2011; Pisetsky DS, et al. 2001; Rus V, et al. 2001). The sex bias of SLE is probably the result of the presence of estrogen receptors on the surface of immune cells and a direct effect on the regulation of cellular responses by these hormones (Rubtsov AV, et al. 2010).

Although the exact etiology of SLE is yet unknown, both environmental and genetic factors contribute to its pathogenesis (Miller et al. 2007; Zandman-Goddard G, et al. 2012). The contribution of environmental factors is believed to be due to robust activation of both the innate and adaptive immune system in individuals genetically predisposed towards autoimmunity. On the other hand, several intrinsic immune abnormalities have been associated with SLE development and severity. Key factors in the establishment of a predisposition towards lupus include aberrancies in clearance of apoptotic and netotic material. Impaired clearance of such material can provide the immune system with a plethora of self-antigens that would otherwise be absent (Bouts YM, et al. 2012). The effect of impaired clearance of cellular debris is further enhanced by the induction of post-translational modifications and cleavage of proteins present in present in apoptotic blebs. Such modifications are believed to generate neo-antigens that are recognized as danger signals and activate the immune system (Cline AM, et al. 2004; Biermann M, et al. 2013).

A.2 B cells in lupus

B lymphocytes are of capital importance for the development and progression of lupus. Since the central role of B cells is antibody secretion and one of the key diagnostic factors and characteristic feature of lupus is the presence of auto-antibodies in patients' serum even years prior to appearance of clinical symptoms, their importance in the disease is obvious (Arbuckle MR, et al. 2003). Most important for the pathogenesis, activity and diagnosis of SLE are the anti-dsDNA antibodies. These antibodies are found in 50-70% of lupus patients, are closely related to disease activity and tissue damage and have been shown to be able to induce inflammation and proteinuria in mouse models after passive transfer (Pisetsky, 2000; Ehrenstein MR, et al. 1995; Gaynor et al. 1997). Other pathogenic auto-antibodies include the anti-NMDA antibodies which target the NR2 subunit of glutamate receptors in the

central nervous system and are considered important for the pathogenesis of certain neuropsychiatric manifestations in a subset of SLE patients, anti-Sm and anti-RNP antibodies targeting components of small nuclear ribonucleoproteins (snRNPs) (Lauvsnes, M.B. et al. 2012; Migliorini P. et al. 2005).

Furthermore, B cells contribute to the pathogenesis of SLE in multiple ways, as demonstrated by MRL/MpJ-Faslpr lupus-prone mice, whose B cells specifically lack antibody secretion ability but are otherwise functional. In these mice, renal and vascular disease were present although in much less severity than controls (Chan OT, et al.1999). Instead, complete deletion of B cells resulted in nullification of the disease in the same mouse strain (Shlomchik et al. 1994). Besides antibody secretion, B cells are also known to present antigens to T cells, secrete pro- and anti-inflammatory cytokines and have lymphogenic properties since the secretion of Lymphotoxin a by B-cells is essential for the formation of tertiary lymphoid tissue.

Moreover, B cells in lupus have been reported to show certain abnormalities such as increased memory and plasma cell expansion, decreased naïve B cell numbers and suppression of inhibitory receptor FcRIIb in memory and plasma cells (Dörner T, et al. 2011; Mackay M, et al. 2006; Su K, et al. 2007).

The significance of B cells in lupus pathogenesis has made B cells an immerging target for therapy development. One of the finest examples of B cell-targeted therapy for SLE is the human monoclonal antibody against BAFF commercially known as Belimumab. Belimumab has shown quite promising results and has been approved by the FDA (Stohl, W. et al. 2012). Moreover, other B-cell targeted therapies focus on B cell depletion with the use of antibodies for B cell specific markers such as CD20 (rituximab), CD22 (epratuzumab), and CD19 (Looney RJ, et al. 2005; Jacobi AM, et al. 2008; Blanc V, et al. 2011).

A.3 Co-signaling receptors

B cell fate is governed by the B-cell receptor (BCR) not only through the stages of antigen-driven activation and differentiation but also through the maturation and selection stages during the developmental stages of the B cell lineage taking place in the bone marrow. Upon antigen stimulation BCR can initiate a plethora of signaling cascades via the activation of a numerous protein tyrosine kinases (PTKs) and phosphatases (PTPs) **(Figure 1)**. Despite its central role, however, the BCR alone is not sufficient for optimal B cell activation and differentiation. A multitude of co-signaling receptors is activated in order to fine-tune and orchestrate the various passages B cells undergo in their journey towards final differentiation.

Most aspects of B cell contribution to inflammation not only in infection but also in the context of SLE are governed by their interaction with T cells through the

arsenal of co-signaling receptors expressed by both B and T cells. These receptors can alter and regulate the magnitude of the immune response, through the manipulation of activation status both on T cells and B cells **(Figure 2)**.

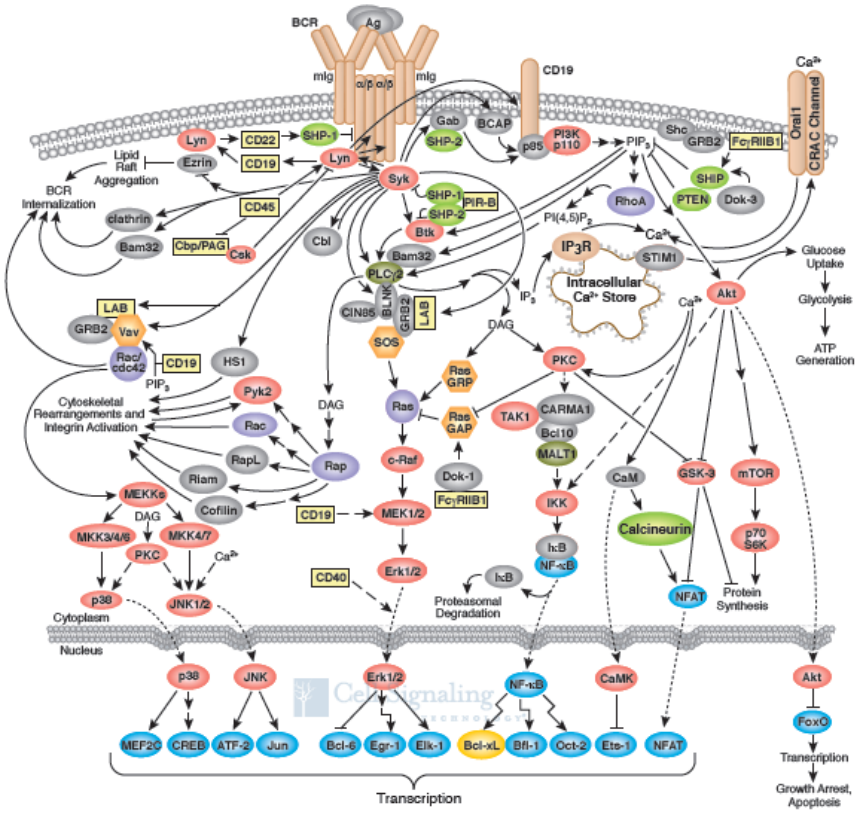

Figure 1. The signaling cascades initiated by the BCR

The co-signaling receptors involved in the regulation of the immune responses are largely divided in two superfamilies, the Immunoglobulin (Ig) superfamily which includes CD28/CTLA-4-B7.1(CD80)/B7.2(CD86), CD40-CD40L, ICOS-ICOSL, PD-1-PD-L1/PD-L2 among other members and the TNF superfamily which contains LTβ/LTβR, HVEM/LIGHT, OX40/OX40L and CD27/CD70 Fas/FasL among others. (Barclay A, 2003; Liossis SN, et al. 2004; Croft M, 2009). Co-signaling receptors can either support (co-stimulators) or reduce (co-inhibitors) cell activation signals deriving from the BCR or TCR (T cell receptor) in T cells. For example, CTLA-4, PD-1, PD-L1, LTβR, Fas are known inhibitors of immune responses, while CD28, CD80, CD86, HVEM, CD27, CD70, ICOS, ICOSL, OX40, OX40L, CD40 and CD40L actively enhance BCR or TCR signaling.

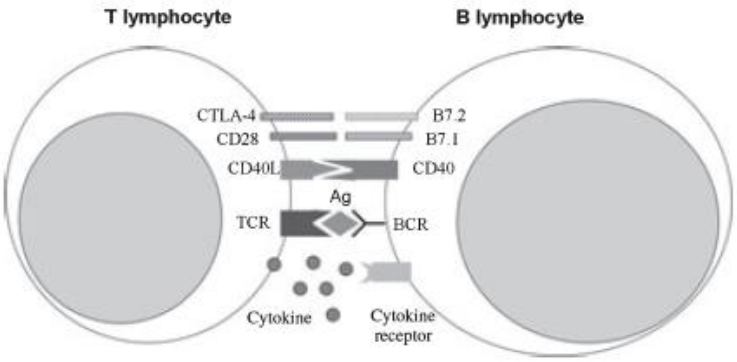

Figure 2. Key co-signaling molecules involved in B-T cell interactions.

A.3.1 HVEM (TNFRSR14)

HVEM (Herpesvirus Entry Mediator), a member of the TNF-Receptor family is a 30,392 kDa transmembrane protein of 283 amino acids. It was discovered by Kwon et al during an expressed sequence database search (Kwon B.S. et al. 1997). Its extracellular N-terminus contains four cysteine-rich domains (CRDs), three of them typical and one lacking one of the three disulfide bonds, characteristic of CRDs (Montgomery RI et al. 1996; Ware CF. 2008). The intercellular c-terminus of HVEM does not contain the characteristic of the TNFR family, death domain resulting in an inability to activate apoptosis directly. However, it does contain the motifs PXWT and IPEEGD that have been shown to recruit and activate members of the TRAF family, TRAF2 and TRAF5 (Marsters SA. 1997; Hsu H. 1997). HVEM is expressed on a variety of immune cell types (B cells, T cells, NK cells, DCs) and organs (brain, liver, lung and kidney) (Montgomery RI et al. 1996; Granger SW et al. 2001; Morel Y. et al. 2001; Duhen T et al. 2004; Harrop JA et al. 1998).

The HVEM signaling network is an unfamiliar case of interaction between the TNFR superfamily and Ig superfamily of receptors/ligands since the five different ligands of HVEM that have been identified so far belong to both families: LIGHT (Lymphotoxin-like excibits Inducible expression and competes with HSV Glycoprotein D for binding to HVEM a receptor expressed by T lymphocytes), LTα (TNFR superfamily), BTLA (B and T Lymphocyte Attenuator), CD160 (Ig superfamily) and glycoprotein D (gD) of HSV virus. Members of the Ig superfamily use the CRD1 domain of HVEM for binding while LIGHT binds in CRD2 and CRD3 domains (Sedy JR, et al**.** 2005; Cai G et al. 2008; Mauri DN, et al.1998; Montgomery RI et al.1996; Sarrias MR, et al. 2000).

As a member of the TNFR family, HVEM requires homooligomerisation for its activation and initiation of signaling events. Engagement of HVEM by either BTLA, CD160 of the Ig superfamily or LIGHT of the TNFR superfamily leads to the

recruitment of TRAF2 and TRAF5 and the activation of NF-kB member, RelA (Hsu et al. 1997; Rooney IA et al. 2000; Cheung TC et al. 2009). Structural studies have shown that LIGHT undergoes homooligomerisation on the cell surface forming a trimeric structure which engages HVEM in a 3:3 stoichiometry (Bodmer et al. 2002) leading in the activation of NF-kB and AP1 (Marsters SA et al 1997). On the other hand contradictory results exist as far as the interaction of BTLA with HVEM is concerned. A light-scattering-based study claims that the engagement of HVEM by BTLA is achieved through a 1:1 complex (Compaan DM et al. 2005). However another study by Cheung et al. (Cheung TC et al. 2009) using FRET [Fluorescent Resonance Energy Transfer-a technique able to estimate the distance between two molecules based on energy transfer between two chromophores (Helms, V. 2008)] has showed that BTLA can form oligomers on native membranes. In the same study there is evidence supporting that BTLA-HVEM interactions may occur under the aid of LIGHT in its soluble form, since it was discovered that sLIGHT can enhance BTLA-mediated NF-kB activation forming a 3:3:3 complex between HVEM/BTLA/sLIGHT (**Figure 3).**

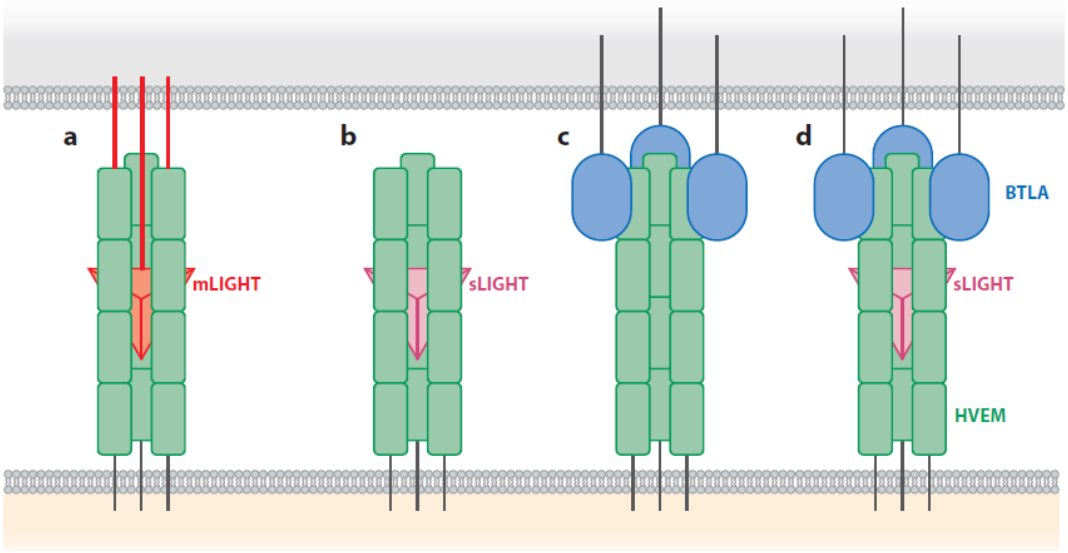

Figure 3. The HVEM/BTLA/LIGHT triumverate interaction complexes

HVEM knockout mice models have demonstrated the importance of HVEM signaling pathways in the regulation of the immune system. HVEM knockout mice have revealed the dominant role of HVEM as a negative regulator of immune responses, since HVEM$^{-/-}$ mice have shown impaired homeostasis of certain T cell subpopulations and DCs (DeTrez C. 2008), increased susceptibility in experimental autoimmune encephalomyelitis (EAE) (Wang Y, et al. 2005) and more robust responses to Concanavalin A (ConA) (Wang, Y. 2005).

A.3.2 BTLA (CD272)

BTLA (B and T Lymphocyte Attenuator) is one of the ligands of HVEM belonging to the Ig superfamily. BTLA was discovered in 2003 by Watanabe N et al. It is a transmembrane glycoprotein of 32,834 kDa containing 289 amino acids (Watanabe N et al. 2003). BTLA signals through three tyrosine motifs in its cytoplasmic tail. The first is an YMN domain that activates PI3K and possibly Grb-2 as well (Gavrieli M. et al. 2006; Alegre ML et al. 2001). The other two belong to the immunoreceptor tyrosine-based inhibitory motifs (ITIMs), are typical components of inhibitory receptors of the immune system and can recruit and activate SH2 domain-containing protein tyrosine phosphatases 1 and 2 (SHP-1 and SHP-2) (Watanabe N. et al. 2003; Han P. et al. 2004; Gavrieli M. et al. 2003). During B cell activation, BTLA has been shown to complex with the BCR components IgM, CD79a and CD79b and attenuate BCR signaling by lowering the levels of phosphorylated Syk. However, since BTLA failed to associate with CD19 and showed no incorporation into lipid rafts it is believed that BTLA signals in the periphery of the BCR complex (Sedy JR. et al. 2005). According to these results BTLA shows a functional similarity to the inhibitory receptors CD22, CD45, CD72 and PIR-B (Maeda, A.et al. 1999; Nitschke, L. 2005; Ravetch J.V. et al. 2000)

BTLA exists in two splice variants. Its major isoform contains the Ig domain; the transmembrane domain and the cytoplasmic signaling tail. However, alternative forms lacking the Ig domain or the transmembrane domain have been reported (Han P. et al. 2004; Watanabe N. et al. 2003). According to Wanatabe et al. BTLA is expressed mainly in cells of the immune system. Most notably, on the T- and B- cell lineages (Han P. et al. 2004). Interestingly, it is expressed on the bone marrow as early as the pro- and pre- B cell stages, as far as the B cell lineage is concerned, and its expression is continued throughout the B cell life (Hurchla MA et al. 2005).

The role of BTLA as an inhibitor of immune responses has been demonstrated through a variety of disease models and the linkage of a SNP of the *btla* gene with increased susceptibility to rheumatoid arthritis (RA). This alteration was enough to change an amino acid residue in the linker region between the two ITIM motifs from leucine to proline. The conformational change in the protein level inhibits the interaction between the ITIM domains and the SH2 domains of SHP-1 and SHP-2 (Lin SC et al. 2006). The functional importance of the BTLA/HVEM interactions in the regulation of the immune system is perfectly demonstrated in BTLA-deficient mice models. These mice show both an increase in the magnitude of their immune responses, such as prolonged airway inflammation upon challenge (Deppong C, et al.2006); an expected feature for an inhibitor knockout and great similarities with HVEM-deficient mice, such as increased succeptibility to MOG-induced EAE, and autoimmune hepatitis-like disease (Deppong C, et al.2006; Watanabe N. et al. 2003; Oya Y, et al**.** 2008). Similar features between HVEM and BTLA knockout mice models are also visible in the function and homeostasis of T-cell subsets (Tao R. et al. 2008). Interestingly enough, BTLA deficiency lead to decreased numbers of total

lymphocytes following a first wave of more robust expansion compared to control lymphocytes (Krieg C. et al. 2007).

A.3.3 LIGHT (CD258)

LIGHT (Lymphotoxin-like shows Inducible expression and competes with HSV Glycoprotein D for binding to HVEM a receptor expressed by T lymphocytes) is a 26,35 kDa TNF family member containing 240 amino-acid residues. As a TNF member, LIGHT forms a homotrimer on the surface of cells (Mauri DN, et al. 1998). LIGHT is a receptor for both HVEM and LTβR and has been shown to interact with soluble Decoy Receptor 3 (DcR3) (Mauri et al. 1998; Bodmer JL, et al. 2002; Yu KY, et al. 1999). Since LTβR is absent in B and T lymphocytes the effect of LIGHT signaling towards these cells is mediated through HVEM only (Wang J, et al. 2005). LIGHT can induce the activation of TRAF2 and TRAF5 acting as a ligand of HVEM. As a receptor, LIGHT has no obvious signaling motifs in its cytoplasmic tail (Marsters SA, et al. 1997; Tamada K, et al. 2002). LIGHT binding to HVEM is mediated through the SRD2 and SRD3 domains of HVEM on the opposite (DARC-gD and BTLA binding site on the TNFR HVEM in CRD1) site of BTLA:HVEM interaction (Rooney IA, et al. 2000; Banner DW, et al. 1993; Whitbeck JC, et al. 2001; Carfi A, et al. 2001). Because of this, the CRD1 domain of HVEM is accessible by BTLA and thus the HVEM:LIGHT:BTLA complex can be formed (Gonzalez LC, et al. 2005). It has been shown that transmembrane LIGHT can be cleaved at amino acid 83 resulting in a 158 long soluble form. sLIGHT retains all the ligand functions of mLIGHT including binding and activating HVEM and enhancing the BTLA:HVEM interaction (Morel Y, et al. 2000; Granger SW, et al. 2001).

LIGHT spectrum of expression contains the spleen, the brain, the lymph nodes, the small intestine, the thymous gland and the appendix (Mauri DN, et al. 1998; Zhai Y, et al. 1998). On a cellular level it is expressed on immature DCs, granulocytes, monocytes, T lymphocytes and B lymphocytes after activation (Morel Y, et al. 2001; Morel Y, et al. 2000; Shi G, et al. 2002; Watts TH, et al. 2005).

$LIGHT^{-/-}$ mouse models have demonstrated the co-stimulatory role of LIGHT in the interactions of the immune system. LIGHT-deficient mice have shown both impaired TCR-mediated T cell activation and longer transplant survival in cardiac allograft survival studies (Ye Q, et al. 2002; Liu J, et al. 2005; Scheu S, et al. 2002; Wang Y, et al. 2005). Interestingly, LIGHT has been thoroughly studied in cancer where it promotes cancer cell death through its interaction with either LTβR or HVEM. LTβR engagement by LIGHT releases cytokines and chemokines leading to lymphocyte accumulation on site, while LIGHT engagement of HVEM expressed by $CD8^{+}$ T cells co-stimulates their activation leading to tumor clearance (Stopfer P, et al. 2004; Yu P, et al. 2004; Tamada K, et al. 2000).

A.4 Aim of the study

Extensive studies have revealed the role of B cells in SLE pathogenesis and adaptive immunity. B cells emerge as key players in immune responses due to their unique properties: antigen presentation, cytokine secretion, regulation of activation of other cell subsets such as T cells, lymphoneogenesis and antibody production. Traditionally, B cells require help from CD4+ T cells for proper activation, clonal expansion and differentiation. Essential elements of this T-cell-provided help are the co-signaling members of the TNF and Ig superfamilies that can deliver both stimulatory and inhibitory signals. HVEM and its ligands, BTLA and LIGHT, hold a unique place since their interactions are the first discovered so far that can functionally link these two superfamilies. The importance of HVEM/BTLA/LIGHT pathways for immune responses has been demonstrated through several mouse models. However little is known for the effect of this signaling network on B cells.

The primary aim of this study was to explore the role of HVEM:BTLA:LIGHT triumvirate co-signaling towards B cell activation in the context of SLE. The study focused on the effect of T cell-expressed HVEM as a co-signaling ligand. HVEM signaling has been extensively studied on T cells but little is known about its effect on B cells. This was addressed by functional assays determining the effect of HVEM on total peripheral blood B lymphocytes. Since B cells undergo excessive remodeling during their activation and differentiation towards plasma cells the effect of HVEM was examined on fractionated naïve and memory B lymphocytes as well. Bearing in mind that there are two HVEM receptors expressed on B cells (BTLA and LIGHT) and that their expression might alter according to their activation status, functional assays were performed with blockade of each individual HVEM receptor was performed in order to dissect the protein interactions occuring during T-B cell interactions.

B. MATERIALS AND METHODS

B.1 Cell isolation

B.1.1 Peripheral Blood Mononuclear Cell (PBMC) isolation

PBMCs were isolated from peripheral blood of healthy donors with Ficoll-Histopaque centrifugation. Whole peripheral blood, no older than 8 hours and supplemented with anticoagulants (heparin or EDTA), was diluted with 1 volume sterile PBS and loaded in 15ml tubes containing Ficoll-Histopaque (at a 2:1 analogy); and centrifuged at 400g/30min/22^{o}C with no brake. The mononuclear cell layer, appearing as a white thin line, was collected with a sterile, plastic transfer pipette in a 50ml tube. The cells were washed with 40ml sterile PBS and centrifuged at 300g/10min/22^{o}C. After aspiration of the supernatant, Red Blood Cell (RBC) lysis took place resuspending the cell pellet in 1ml sterile dH_2O for 25sec and subsequently adding 1ml of sterile NaCl solution in dH_2O (1.8%). After the RBC lysis the cells were washed with 40ml PBS and centrifuged at 300g/10min/22^{o}C.

After the second wash with PBS, the cell pellet was transferred in 15ml tube, washed with 10ml MACS buffer and centrifuged at 200g/15min/22^{o}C for 3 or 4 times until the platelets in were removed. PBMC and platelet numbers were calculated using light microscopy in a volume of the resuspended cells diluted in Trypan Blue and placed in a Neubauer heamocytometer.

Reagents and Instruments

1. Ficoll-Paque™ PLUS (GE Healthcare Life Sciences, Uppsala, Sweden)

2. Phosphate-buffered Saline (PBS)

3. MACS buffer (PBS, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS BSA Stock Solution 1:20 with AutoMACS™ Rinsing Solution)

4. Trypan Blue (Gibco, Life Technologies, Carlsbad, California, USA)

5. Pasteur pipettes (Sarstedt, Nuembrecht, Germany)

6. Neubauer haemocytometer (Assistent, Germany)

7. Light microscope (Nikon TMS, Tokyo, Japan)

B.1.2 B cell isolation

Isolated peripheral blood mononuclear cells, diluted in MACS buffer, were incubated with appropriate volume of Biotin antibody cocktail for 10 minutes at a temperature of 2-8^{o}C. After incubation the cells were diluted in additional MACS

buffer and the appropriate volume of Anti-Biotin Microbeads was added. Cells were incubated in the refrigerator (2-8^{o}C) for 15minuites. After incubation the cells were diluted at 500 μl final volume with MACS buffer and the suspension was applied onto a MACS MS column, properly placed in the magnetic field of a MiniMACS™ Separator. One washe with 500 μl MACS buffer were performed prior to the removal of the column from the separator. The flowthrough of the column was collected in a 15ml tube. A portion of the cells were mixed with Trypan Blue and the suspension was placed onto Neubauer haemocytometer in order to be counted by light microscopy. Purity (B cells/total separated live cells) was evaluated by flow cytometry.

Reagents and Instruments

1. MACS buffer (PBS, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS BSA Stock Solution 1:20 with AutoMACS™ Rinsing Solution)

2. B cell isolation kit II (containing B cell Biotin-Antibody cocktail and Anti-Biotin Microbeads) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)

3. Trypan Blue (Gibco, Life Technologies, Carlsbad, California, USA)

4. MACS MS columns and plungers (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)

5. MiniMACS™ Separator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)

6. Neubauer haemocytometer (Assistent, Germany)

7. Light microscope (Nikon TMS, Tokyo, Japan)

B 1.3 T helper ($CD4^{+}$) cell isolation

Isolated PBMCs, diluted in MACS buffer, were incubated with appropriate volume of CD4 Microbeads for 15 minutes at a temperature of 2-8^{o}C. After one wash with MACS buffer, the cells were centrifugated at 300g/10min/4^{o}C and resuspended with 500 μl MACS buffer and the suspension was applied onto a MACS MS column, properly placed in the magnetic field of a MiniMACS™ Separator. 3 washes with 500 μl MACS buffer were performed prior to the removal of the column from the separator. The column was then placed into a suitable collection tube and 1 ml MACS buffer was added onto the column. The magnetically labeled $CD4^{+}$ T cells were flushed out by firmly pushing the plunger into the column. Cells were mixed with Trypan Blue and the suspension was placed onto Neubauer haemocytometer in order to be counted by light microscopy. Purity was evaluated by flow cytometry.

Reagents and Instruments

1. MACS buffer (PBS, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS BSA Stock Solution 1:20 with AutoMACS™ Rinsing Solution)

2. CD27 Microbeads (Microbeads conjugated to monoclonal anti-human CD27 antibodies) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)

3. Trypan Blue (Gibco, Life Technologies, Carlsbad, California, USA)

4. MACS MS columns and plungers (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)

5. MiniMACS™ Separator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)

6. Neubauer haemocytometer (Assistent, Germany)

7. Light microscope (Nikon TMS, Tokyo, Japan)

Note: The exact same protocol was used for the isolation of naïve and memory B cells (characterized as $CD27^-$ and $CD27^+$ respectively) from freshly isolated total B cells. In this case, $CD27^+$ B cells were flushed from the column and $CD27^-$ B cells were collected in the flowthrough of the column.

B.2 Functional assays

B.2.1 B cell cultures

For functional assay cultures, total, naïve or memory B cells were cultured for 5 days in 96-well plates at a density of 100.000 cells/well in DMEM medium supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, 100μg/mL streptomycin, 10 mM HEPES and 1 mM sodium pyruvate. The wells were pre-coated with with recombinant human HVEM-Fc chimeric protein at two concentrations: [(2 μg/ml) and (10 μg/ml)] or with (10μg/ml) of human IgG1 Isotype control in PBS. The wells were left at 4oC overnight. After 2 washes with fresh PBS, the cells were placed in the wells and stimulated with 5 μg/ml goat F(ab')2 anti-human IgM+IgG, 1 μg/ml anti-CD40 and recombinant human IL-21.

Reagents and Instruments

1. DMEM medium (Gibco, Life Technologies, Carlsbad, California, USA)

2. Fetal Bovine Serum (FBS) (Gibco, Life Technologies, Carlsbad, California, USA)

3. Penicillin/Streptomycin Solution 100x (Biosera, Boussens, France)

4. AffiniPure $F(ab')_2$ Fragment Goat Anti-Human IgG + IgM (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA)

5. Anti-human CD40 Functional Grade Purified (eBioscience, Inc., San Diego, California, USA)

6. Recombinant human IL-21

7. Recombinant Human HVEM/Fc Chimera (R&D Systems, Inc., Minneapolis, MN, USA)

8. Human IgG1

9. Costar® Corning® 96-well cell culture clusters, round-bottomed (Corning Inc., NY, USA)

B.2.2 CFSE assay

Purified total, naïve or memory B cells (as indicated) were resuspended in 1 ml pre-warmed PBS/BSA 0.1% /maximum 5 x 10^6 cells in a 15-ml falcon tube. In this tube, 1 μl of CFSE (5 mM)/10^6 cells was placed in a drop of 100 μl PBS. The tube was covered immediately with foil and the cell suspension was mixed vigorously. After incubating the cells at 37^{o}C for 10 minutes, 5 ml ice-cold PBS/FBS 5% were added and the cells were centrifuged at 1400 rpm for 10 minutes at room temperature. Cells were resuspended in complete medium in suitable volume and added to the culture. Cell proliferation was measured with flow cytometry after 3 days of culture.

Reagents and Instruments

1. Phosphate-buffered Saline (PBS)

2. Fetal Bovine Serum (FBS) (Gibco, Life Technologies, Carlsbad, California, USA)

3. Bovine Serum Albumin Fraction V (7,5%) (Gibco, Life Technologies, Carlsbad, California, USA)

4. CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, Carlsbad, California, USA)

B.2.3 T cell-B cell co-cultures

For functional assay cultures, total B cells were co-cultured with $CD4^+$ T cells for 7 days in 96-well plates at a density of 200.000 cells/well (1:1 ratio) in DMEM medium supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, 100μg/mL streptomycin, 10 mM HEPES and 1 mM sodium pyruvate. T cells were stimulated with soluble anti-human CD3 (5μg/ml) and soluble anti-human CD28 (1μg/ml). $CD4^+$ T cells were treated with: FcR Blocking Reagent for 10min/4^{o}C Mitomycin C (30μg/ml/10^7 cells) for 30min/37^{o}C Anti-human HVEM (20μg/ml or 40μg/ml) for 30min/4^{o}C prior to culture. In some conditions instead of $CD4^+$ cell pretreatment, B cells were treated with anti-human BTLA (10 μg/ml or 20 μg/ml) for 30min/4^{0}C prior to culture.

Reagents and Instruments

1. DMEM medium (Gibco, Life Technologies, Carlsbad, California, USA)

2. Fetal Bovine Serum (FBS) (Gibco, Life Technologies, Carlsbad, California, USA)

3. Penicillin/Streptomycin Solution 100x (Biosera, Boussens, France)

4. Anti-human CD3 Functional Grade Purified, Clone: OKT3 (Affymetrix, eBioscience, Inc., San Diego, California, USA)

5. Anti-human CD28 Functional Grade Purified, Clone: CD28.2 (eBioscience, Inc., San Diego, California, USA)

6. Purified anti-human CD270 (HVEM, TR2), Clone:122 (Biolegend, San Diego, California, USA)

7. anti-BTLA (human), mAb (6F4) (AdipoGen, Inc., Sondo-dong, Yeonsu-gu, Incheon, South Korea)

8. Mitomycin C from Streptomyces caespitosus-powder, BioReagent, suitable for cell culture (Sigma – Aldrich, Saint Louis, Missouri, USA)

9. FcR Blocking Reagent (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)

10. Costar® Corning® 96-well cell culture clusters, round-bottomed (Corning Inc., NY, USA)

B.3 FACS staining protocol

Cells (either freshly isolated or cultured) were washed once with PBS by undergoing centrifugation at 300g/10min/22^{o}C. Next, they were stained with appropriate volume of monoclonal anti-human antibodies according to the manufacturer's protocol in different combinations depending on the experimental settings. After incubating the cells for 30 minutes at 4^{o}C in the dark, the cells were washed with 1 ml PBS/FBS 5% and centrifuged at 300g/10min/4^{o}C and resuspended in 300μl PBS/FBS 5%. Then, the cells were subjected to flow cytometry on a Cytomics FC 500 flow cytometer. Analysis was performed with Flowjo and GraphPad Prism 5 software.

Reagents

- Anti-human CD19, CD138, LIGHT (Biolegend, San Diego, California, USA)
- Anti-human CD27, CD86, HLA-DR (eBiosciece, Inc., San Diego, California, USA)
- Anti-human IgD (BD Pharmingen, San Diego, California, USA)
- Anti-human CD38 (Beckman Coulter, Brea, California, USA)

B.4 IgG Detection in Culture supernatants

IgG detection in culture supernatants was performed with Easy-Titer Human IgG (H+L) Assay Kit by Thermo Scientific (Rockford, IL, USA).

B.5 Participants

Four (4) active SLE patients diagnosed according to the American College of Rheumatology 1982 criteria and followed up at the Rheumatology Department of the University Hospital of Heraklion, Crete were studied. Healthy age- and sex-matched volunteers from the Department of Transfusion Medicine of the University Hospital of Heraklion, Crete served as controls. All subjects gave written informed consent prior to study. The study has been approved by the Ethics Committee of the University Hospital of Heraklion

C. RESULTS

C.1 Expression of HVEM ligand LIGHT in resting and stimulated B cells

Previous data from our lab have shown that BTLA is upregulated in B cells after BCR stimulation compared to baseline levels in samples from healthy donors. Furthermore, higher levels of BTLA are present in B cells from SLE patients (Zampoulaki A, 2013). Furthermore, LIGHT has been reported to be absent in naïve B cells and be expressed upon stimulation (Morel Y, et al. 2001; Morel Y, et al. 2000; Shi G, et al. 2002; Watts TH, et al. 2005). Furthermore, as far as autoimmune disorders are concerned, LIGHT has been reported to be upregulated in the patients with rheumatoid arthritis (Kang YM. et al. 2007). Thus, we decided to evaluate the expression of LIGHT in healthy donor-derived B cells before and after BCR stimulation. Additionally we evaluated the levels of LIGHT expression in B cells from SLE patients.

Figure 4 shows the levels of LIGHT expression in B cells from healthy donors and SLE patients, both in terms of cell percentage and MFI. In healthy donors, LIGHT expression in naïve B cells was found to be minimum with maximum induction after 3 days of stimulation, while its expression on memory B cells was upregulated. Consistent with the pattern of BTLA and the induction of its expression after stimulation, LIGHT is expressed more robustly in B cells from SLE patients compared to healthy donors. As shown in figure 4, this upregulation of LIGHT is detectable in both naïve and memory B cells. Since HVEM is upregulated as well in $CD4^+$ T cells from SLE patients (Zampoulaki A, 2013) it is very likely that the so-stimulatory axis formed by HVEM and its ligands BTLA and LIGHT is contributing in the activation status of B cells both in the context of SLE and under inflammatory conditions.

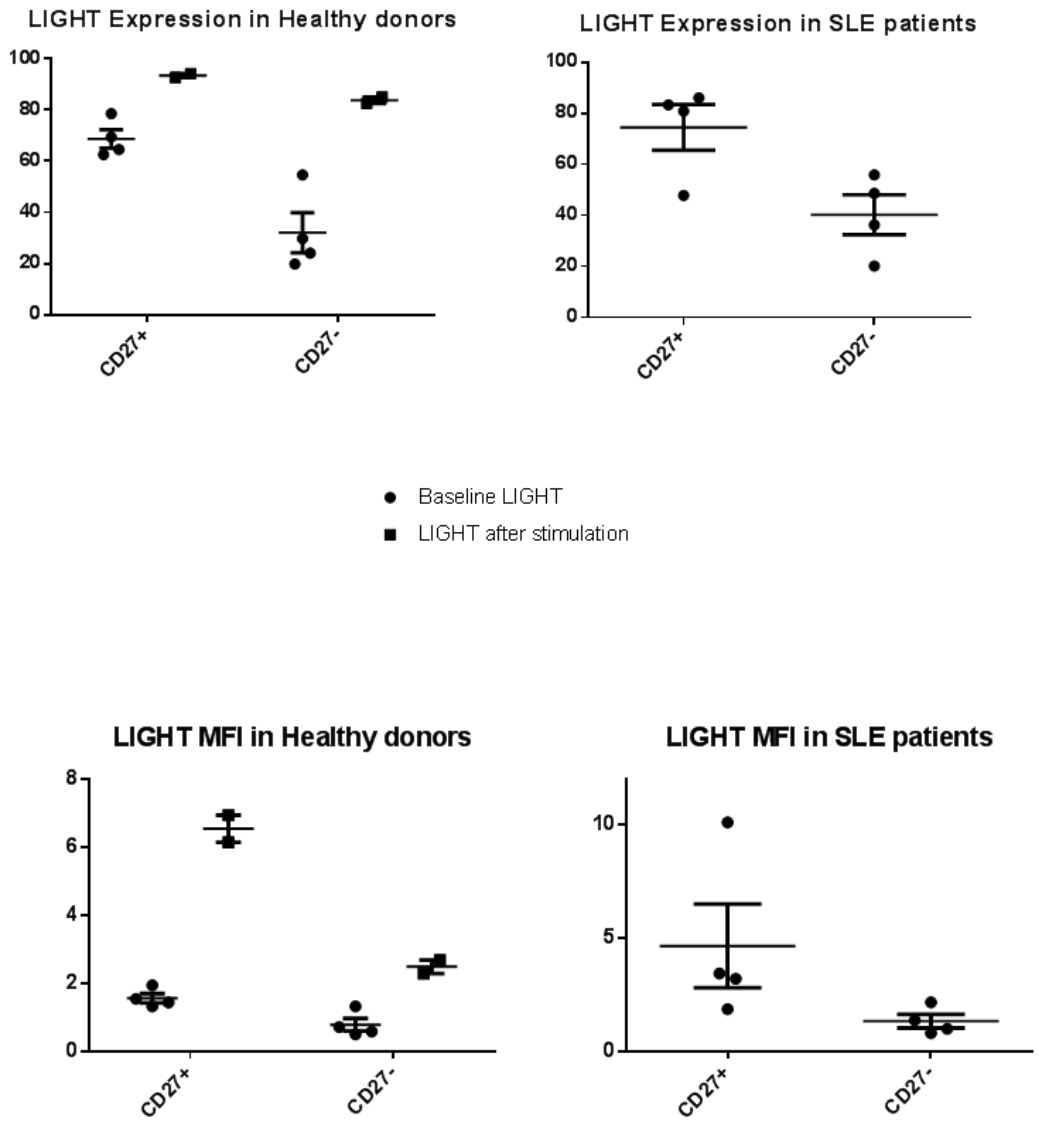

Figure 4. Expression levels of LIGHT in B cells from healthy donors and SLE patients (n=4). The right panel shows the expression levels of LIGHT in Healthy donors prior and after activation. The loft panel shows the expression of LIGHT in SLE patients. Cells from both healthy donors and SLE patients were divided in two major subsets of B cells; naïve ($CD27^-$) and memory ($CD27^+$) cells. The cells were cultured for 3 days. (n=3, n=2 for unstimulated and stimulated levels respectively)

C.2 Effect of HVEM signaling in the activation status of total B cells

To determine the effect of HVEM signaling in B cells after BCR stimulation, B cells were isolated from peripheral blood and cultured in pre-coated 96-round well plates, stimulated with anti-IgM/anti-CD40/IL-21 ± HVEM-Fc. After 5 days of culture the activation status of B cells was determined by Flow Cytometry using various B cell activation markers. Since there seems to be a parallel upregulation of HVEM in $CD4^+$ T cells and LIGHT in B cells (while BTLA is constitutively expressed in B cells), there seems to be a contribution of HVEM and its ligands on the activation of B cells. Furthermore, the co-signaling from the HVEM-BTLA-LIGHT triumvirate seems to take place during the T-B cell interactions during the antigen-driven B cell activation route.

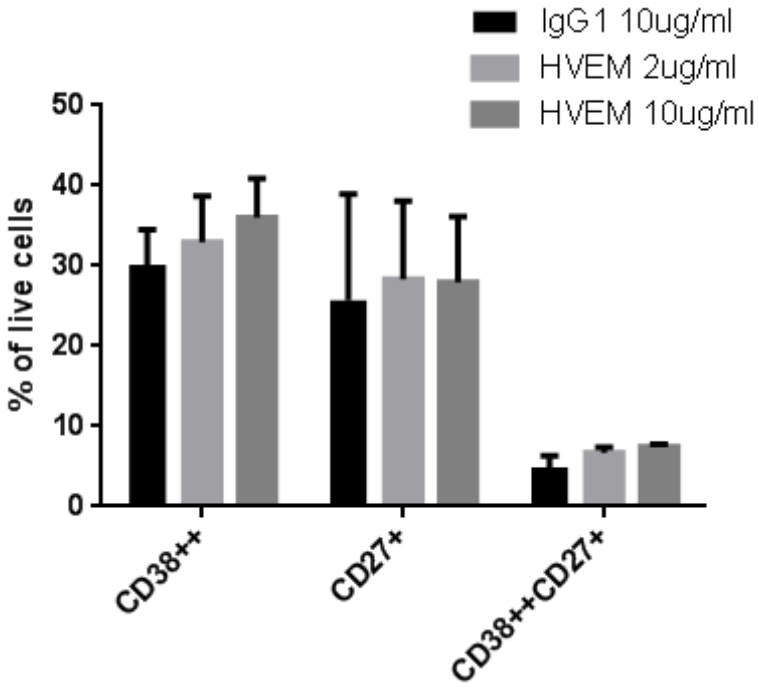

Figure 5. Expression of activation markers on B cells from healthy donors after 5 days of culture. Cells were stimulated with a-IgM+a-IgG (5ug/ml), a-CD40 (1ug/ml) and IL-21 (50ng/ml) (n=2)

In order to simulate these interactions under ex vivo culture conditions B cells were activated with anti-human IgM+IgG, anti-CD40 and IL-21 in order to mimic all three signals delivered by T cells. Furthermore, the wells of the culture were pre-coated with the chimeric protein HVEM-Fc carrying the Fc segment of human IgG1.

As shown in Figure 5, HVEM signaling towards B cells seems to slightly increase the expression of activation markers and contribute to the differentiation of B cells after stimulation.

C.3 Effect of HVEM signaling in the activation status of naïve ($CD27^-$) and memory ($CD27^+$) B cells

So far our results show that HVEM has a role in both the activation and differentiation of B cells and their survival. Given the fact that the apart from the surface markers, the cytoplasmic milieu of naïve and memory B cells is completely different it was quite interesting to evaluate the effect of HVEM signaling towards each B cell subpopulation using the same experimental setup as before.

We evaluated the robustness of $CD27^-$ cells activation by measuring the induction of the activation marker CD27 (Figure 6). HVEM co-stimulated cells had a higher induction of CD27, indicating that the robust activation observed in total B cells after HVEM signaling was, at least partially, due to the effect of HVEM on naïve B cells. Moreover, the effect seems to be dose-dependent since cells cultured in pre-coated wells with the low HVEM-Fc concentration (2ug/ml) had lower levels of CD27 compared with the cells cultured with the high HVEM concentration (10ug/ml) (Figure 6). These results show that HVEM signaling is contributing to the transition of naïve B cells towards memory cells.

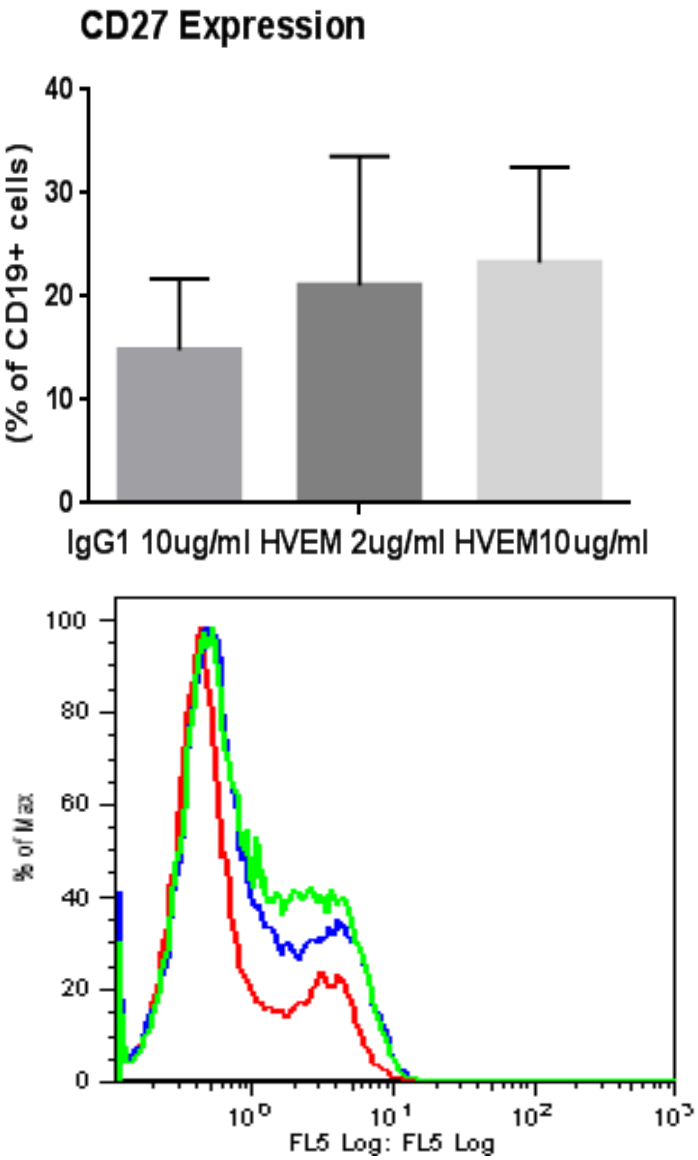

Figure 6. CD27 expression in naïve B cells ($CD19^+CD27^-$) after 5 days of culture. The histograms below show the expantion of $CD27^+$ population in the cell culture. Isotype control IgG1 (10ug/ml) is represented in red, HVEM-Fc (2ug/ml) in blue and HVEM-Fc (10ug/ml) in green. (n=2)

Similarly, memory B cells were stimulated with anti-IgM/anti-CD40/IL-21 ± HVEM-Fc and their activation status was evaluated using the expression of activation marker CD38, plasma cell marker CD138 and IgD. In accordance with our results so far, HVEM co-stimulated cells showed a more differentiated phenotype compared to control. Intriguingly enough, HVEM at a lower concentration (2ug/ml) seemed to have a more robust effect compared with the high concentration of 10ug/ml (Figure 7). Although the reason for this is still unclear, the results demonstrate that both major subpopulations of B cells are positively affected by HVEM regarding their activation and differentiation status.

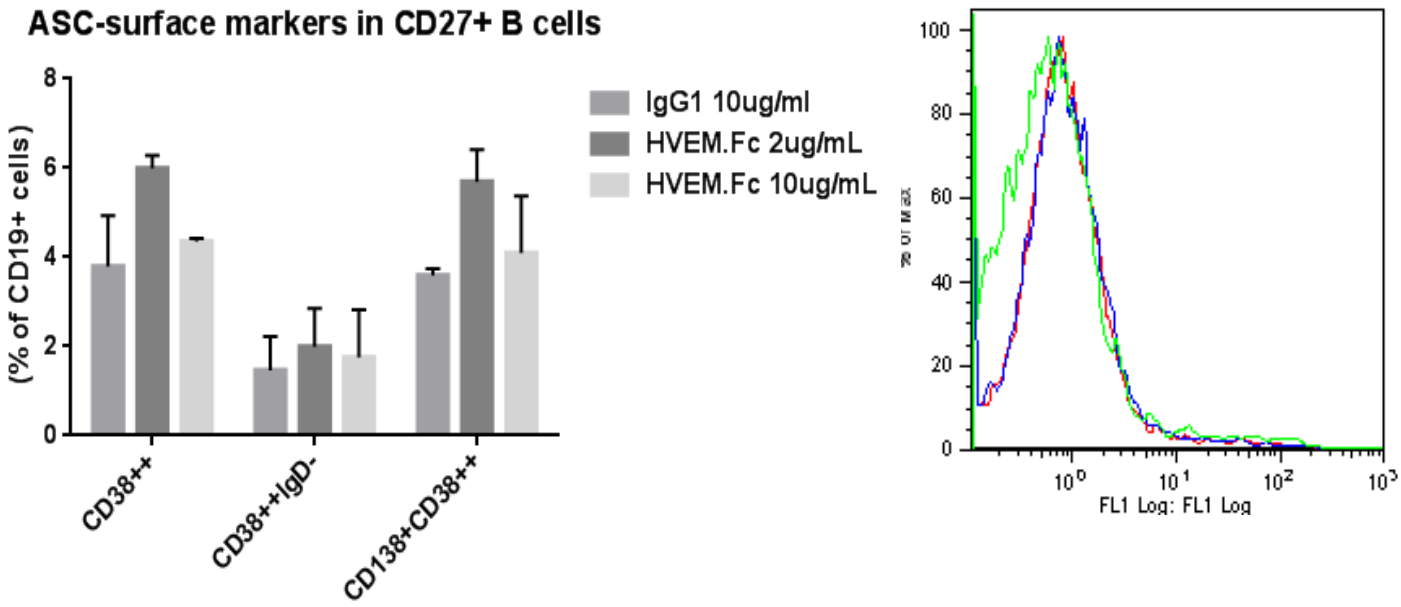

Figure 7. Effect of HVEM on the differentiation of memory B cells upon BCR activation. The left panel shows the expression of CD38 alone and together with other markers associated with the plasmablast or plasma cell phenotype. The left panel shows the expression of IgD marker, the absence of which signifies class-switch recombination. IgG1 co-stimulated cells are shown in red, HVEM co-stimulated cells shown in blue (2ug/ml) and green (10ug/ml). (n=2)

C.4 Effect of HVEM signaling on B cell survival and proliferation

Bearing in mind that during their activation and differentiation B cells undergo several cycles of proliferation and a significant percentage of the cells undergo apoptosis, the results presented so far raise a question regarding the nature of HVEM involvement in B cell activation and differentiation. On one hand, there is the possibility of active induction of B cell differentiation by HVEM, while on the other hand, the shift towards a more robust activation status suggested by our results could

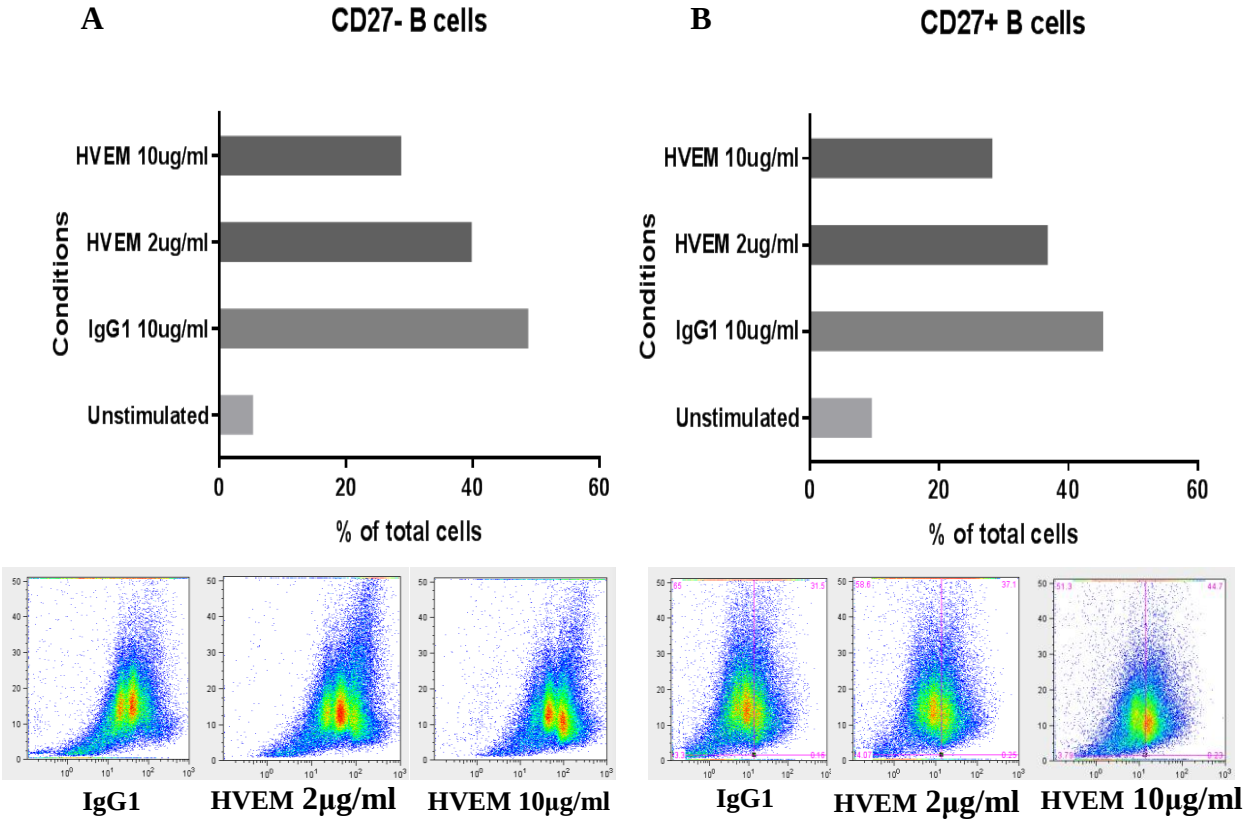

Figure 8. (A) Percentage of proliferated cells after 3 days of culture in naïve B cells. (B) Percentage of proliferated cells after 3 days of culture in memory B cells. The upper panels show the percentages of divided cells, while the ower panels show raw flow cytometry results for each of the B cell subpopulations. Cells were stained with CFSE prior to culture and cell proliferation was estimated as loss of CFSE fluorescence. (n=3 for both sets of experiments)

be the outcome of HVEM-mediated anti-apoptotic effect or HVEM-driven proliferation. In order to understand better which process is the target of HVEM-mediated signaling, proliferation and survival assays were carried out using purified naïve and memory B cells characterized by the expression of CD27, as $CD27^-$ and $CD27^+$ respectively.

As shown in Figure 8, HVEM signaling has comparable effect in both naïve and memory B cells following BCR stimulation. In both cases cells cultured in HVEM-Fc pre-coated wells underwent proliferation to lesser extend compared with IgG1-treated control. B cell proliferation was estimated as loss of CFSE fluorescent intensity in divided cells.

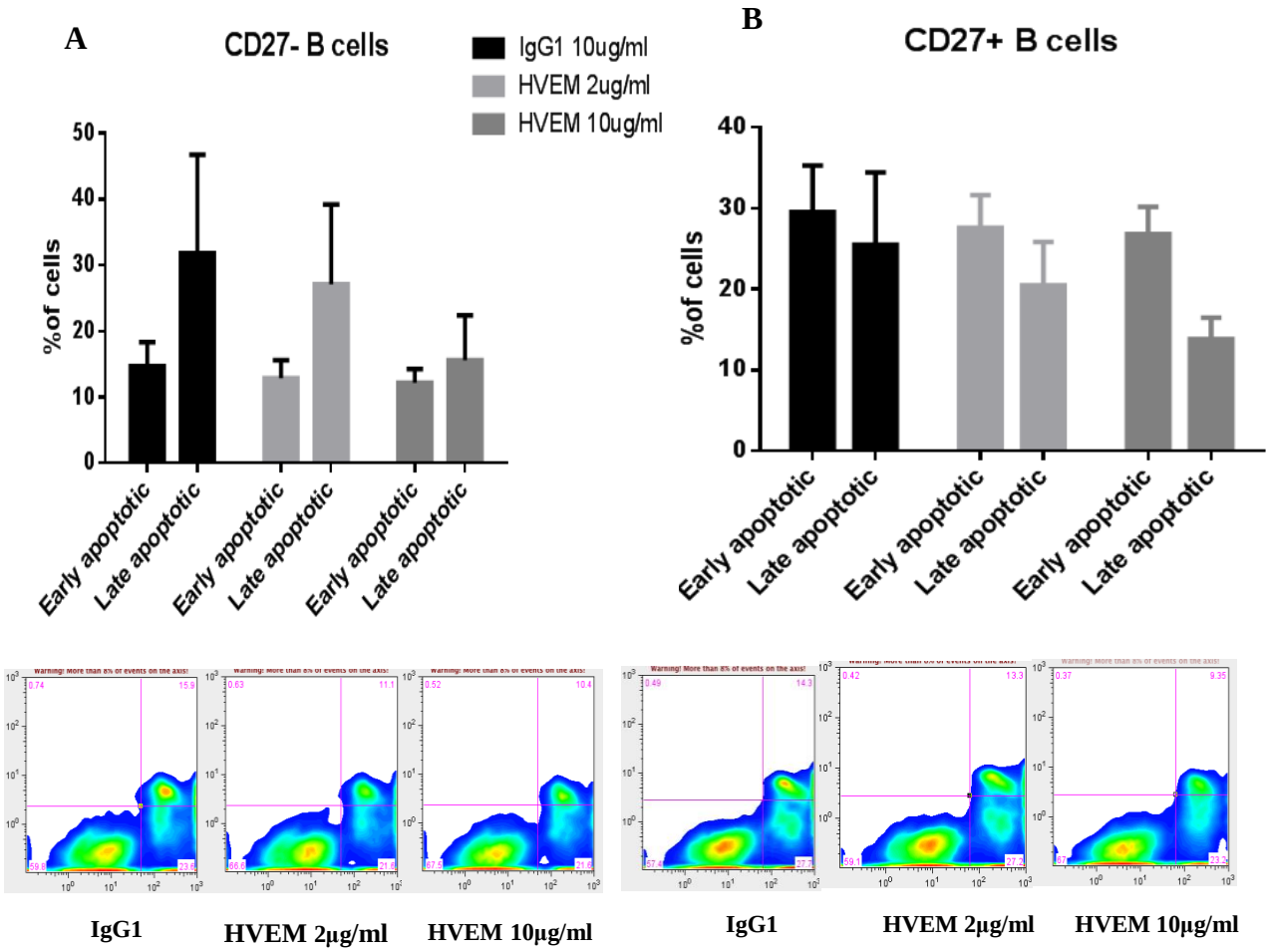

Figure 9. The effect of HVEM signaling towards (A) naïve and (B) memory B cells after 3 days of BCR stimulation. Apoptotic cells were quantified with Annexin V staining. The figure shows the percentage of early apoptotic (Annexin V^+7AAD^-) and late apoptotic (Annexin V^+7AAD^-) cells in the culture. The upper panels show collective results from 3 experiments (n=3). The lower panels show representative figures from Flow Cytometry.

Since culture duration was 3 days, and LIGHT expression on B cell surface was estimated to peak after 3 days of culture (Figure 4), the results suggest that HVEM-mediated BTLA activation is most likely responsible for this effect. Nonetheless, the data show that the HVEM-mediated upregulation of activation B cell markers observed above (Figures 5, 6, 7) is not a result of stronger proliferation

Next, the effect of HVEM signaling on naïve and memory B cell apoptosis was evaluated. As seen in Figure 9, HVEM signaling reduces the apoptosis rate of both B cell subtypes. Interestingly enough, early apoptotic cell percentages seem unaltered while only the late apoptotic cells are reduced.

Collectively these data show that HVEM has an anti-apoptotic role towards both naïve and memory B cells when acting as a ligand. Although they lower the proliferation rate of cells, HVEM-delivered signals also reduce apoptosis, which is quite robust when B cells are activated and differentiate. Furthermore, the effect seems to be dose-dependent as the high concentration of HVEM-Fc tested had a more profound effect on cells that the low one.

C.5 Blocking of HVEM and its ligand BTLA in T cell-B cell co-cultures.

The results so far have shown a dual role of HVEM in the co-stimulation of B cells. Since the experimental setups used so far mimicked $CD4^+$ T cell stimulation of B cells, we decided to evaluate the role of HVEM in T cell- B cell co-cultures using blocking monoclonal antibodies targeting HVEM and BTLA. T cells were stimulated with anti-human CD3 and anti-human CD28. HVEM on T cells and/or BTLA on B cells were blocked prior to culture with specific anti-HVEM (clone 122) and anti-BTLA (clone 6F4) and the activation of B cells was evaluated through the expression

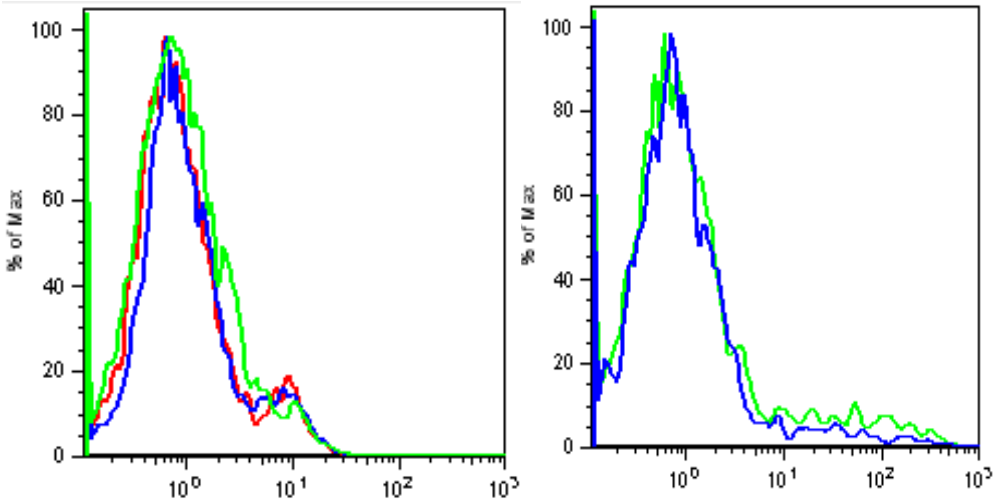

Figure 10. HVEM blockage in T cell- B cell co-cultures. The histograms show the expression of activation marker CD27 on total B cells. Control is represented with green, anti-HVEM (20μg/ml) in blue and anti-HVEM (40μg/ml) in red. No significant differences can be observed. The figure is representative of 2 individual experiments

of activation marker CD27. The expected result in accordance with what has been observed so far, would be a reduction in CD27 expression on B cells that didn't receive HVEM signaling. However, there were no significant differences between culture conditions (Figure 10). Additionally, similar results were obtained when one of the receptors of HVEM on B cells, BTLA was blocked. BTLA is a well known attenuator of immune responces therefore, blockade of its interaction with HVEM should enhance B cell activation. Yet the results show no difference between B cells treated with anti-BTLA and untreated cells (Figure 11). Despite this, these experiments may have to be repeated in purified naïve and memory B cells.

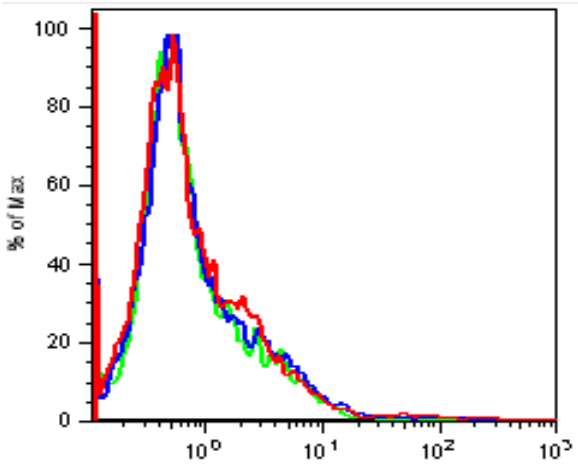

Figure 11. CD27 expression in B cells after BTLA blockage. B cells were co-cultured with $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28. Control (untreated) cells are represented in green, anti-BTLA (10μg/ml) treated cells in blue and anti-BTLA (20μg/ml) treated cells in red.

C.6 IgG detection in culture supernatants

To further assess the differentiation of B cells we measured the titer of IgG secreted in culture medium. Culture supernatants from the functional experiments shown above were stored at -80^{o}C in order to preserve unaltered any secreted products such as antibodies and cytokines. For the detection and quantification of IgG levels in the samples, Easy-Titer Human IgG (H+L) Assay Kit was used by Thermo Scientific. Unfortunately, the sample dilution proposed by the kit was too low and exited the range of standards (Table 1).

Standards (ng/ml)	500	250	125	32.5	31.2	15.6
Mean OD	0.99	1.06	1.15	1.25	1.30	1.32
Samples	Unstimulated	Untreated	rIgG	hIgG	a-BTLA	
Mean OD2	1.38	1.35	1.35	1.36	1.36	

Table 1. Results from IgG detection assay showing the mean OD of 5 culture samples and standards loaded in triplicates. Unfortunately the samples' OD is exceeds the range of Standard OD which means that IgG titer in the diluted samples was lower than 15.6ng/ml

D. DISCUSSION

B cells have multiple roles in immune responses not only through antibody secretion but also through antigen presentation, lymphogenesis and cytokine production. In the context of SLE, B cells are the cornerstone for the development of the disease, as demonstrated by the lack of nullification of lupus in lupus-prone mouse models after B cell depletion (Shlomchik et al. 1994). Furthermore, despite its wide diversity of symptoms; auto-antibody detection in patients' serum is enough for the diagnosis of SLE (Arbuckle MR, et al. 2003). The coupling of BCR signaling with co-signaling receptors is the main force driving B cell activation and differentiation. BCR co-stimulation takes place at the follicles of GCs during T cell-B cell interactions. In this study we focused on the role of HVEM, a member of the TNFR superfamily of co-signaling receptors which is expressed on the surface of both B cells and T cells. HVEM signaling towards B cells is mediated through interactions with its receptors, BTLA and LIGHT.

We first evaluated the expression levels of LIGHT in both naïve ($CD27^-$) and memory ($CD27^+$) B cells from healthy donors and SLE patients. We confirmed partially the expression kinetics reported in the literature, since activated (memory) B cells showed high expression of LIGHT while naïve B cells showed lower levels of expression. Contrary to previous studies we did not find LIGHT undetectable in naïve B cells although this might reflect drawbacks in the estimation of naïve B cells used. Further analyses using early activation markers such as CD80, CD86 might smooth these differences. Additionally, LIGHT expression levels in SLE patients although higher, showed the same pattern as in healthy donors. BTLA, has been reported to be constitutively expressed on B cells (Hurchla MA et al. 2005) while previous data from our lab have shown that BTLA expression is also elevated in B cells from SLE patients (Zampoulaki A, 2013). Taken together these results indicate that HVEM signaling might be contributing to the activation of B cells in SLE.

In order to evaluate the role of HVEM in the activation of B cells, we performed activation assays where, total, naïve and memory B cells were stimulated while receiving co-stimulatory signals from HVEM. HVEM signaling showed to be beneficial for the activation and differentiation of all B cell subsets as demonstrated by the expression of activation markers CD27 and CD38 (Figures 6, 9, 10). Although in total B cells the difference between controls and HVEM-stimulated cells is modest, the pattern of CD27, CD38 induction is following the same trend in all B cell subsets. Considering culture duration and the fact that the co-inhibitor BTLA is the only available HVEM receptor on the surface of naïve B cells for the first 48 hours of activation, it is quite possible that the small differences observed are the aftermath of BTLA-mediated attenuation of B cell activation.

Third, we confirmed the pro-survival effect of HVEM in B cells after BCR activation with survival assays. Both naïve and memory B cell subsets had improved survival, demonstrated as a reduction in the percentage of late apoptotic cells, when

co-stimulated by HVEM (Figure 8). Additionally, HVEM signaling seems to have an anti-proliferative effect in naïve and memory B cells as shown in Figure 7.

Blocking of HVEM and its receptor BTLA in T cell- B cell co-cultures did not show any differences in the state of B cell activation. However, since HVEM is expressed by both T cells and B cells, HVEM blocking was performed prior to culture so it is quite possible that due to protein recycling, the blocked molecules of HVEM were replaced shortly after with newly synthesized ones. Alternatively, assays where B cells will be placed in HVEM pre-coated culture wells in the presence of soluble blocking antibodies for BTLA and LIGHT might determine which of the receptors of HVEM is responsible for the observed changes. Furthermore, culture setups using CD40L and HVEM transfected cell lines might simulate even better the interactions between T cells and B cells in vivo.

In conclusion, our results so far show that the HVEM-BTLA-LIGHT triumvirate has a dual role during T cell-mediated B cell activation: during the first days of activaton HVEM attenuates B cell activation. Moreover, HVEM signaling has a clear cell protective effect by lowering the speed of proliferation and the magnitude of apoptosis in B cells. Considering the time frame in which these phenomena take place it is quite possible that they are mediated through BTLA which is a well known attenuator of immune responses and is constitutively expressed by B cells. However, after the first 48- 72 hours of activation and as BCR signaling continues to drive B cell differentiation and alter the expression of membrane and cytoplasmic signaling molecules in B cells, HVEM seem to switch roles and act as a co-stimulator. Whether this role switching is performed via LIGHT, whose expression peaks at that point or it is still mediated through BTLA signaling to an altered cell environment, remains to be seen.

As SLE therapies switch from traditional to more sophisticated strategies, B cells have become a major target. As the search for new therapies continues, important players of B cell activation will be targeted for therapy. HVEM could be an emerging target in the future provided that research continues on the topic of its fascinating role in the co-stimulation of B cells.

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